Supplemental Information
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SUPPLEMENTAL FIGURE LEGENDS

Figure S1, related to Figure 1

A. Biliary cells (BC) show enrichment of Yap and Hippo pathway targets. Hepatocytes (Hep) and biliary cells were isolated and relative mRNA expression of noted genes/categories were quantitated via qPCR. Hepatocytes are normalized to one and BC expression is quantitated as a ratio to its expression. Data represent the average of 3 biological samples +/- SEM. B. Experimental design for biliary-specific Yap overexpression. Mice with Ck19-CreER and TetOYap alleles are given tamoxifen to permit tetracycline-inducible Yap expression. Doxycycline is then administered to activate expression of Yap. Examples of Yap overexpression in the bile duct utilizing the Ck19-CreER mice at various time points as indicated (200x magnification). C. AAV-Cre does not infect biliary cells of the mTmG reporter mouse. Confocal image of a representative bile duct of an mTmG reporter mouse given PBS or 10^11 pfu AAV-Cre (Cre) two weeks after infection. Asterisk indicates position of the bile duct. Table below indicates the total number of portal areas examined from three mice and the percentage of biliary cells that were positive for mTomato. D. 0.2% of BCs are infected by AAV-cre. Cartoon depicting strategy for analyzing AAV-Cre infectivity in hepatocyte versus biliary compartment by enzymatic digestion and FACS analysis. Representative linear YFP FACS plot is shown below for hepatocytes and biliary cells. Line depicts the gate defined as YFP+ cells in both cell populations. The number above depicts the number of positive cells averaged from three experiments. E. Microscopic observation and FACS analysis of organoids derived from a PBS or AAV-Cre infected Rosa26-lsl-tdTomato mouse. Representative 40x field shows rare tdTomato+ organoids in AAV-Cre infected mouse. Below, representative linear Tomato FACS plot is shown control and AAV-Cre infected organoids. Line depicts the gate defined as Tomato+ both cell populations. The number above depicts the number of positive cells averaged from three experiments. F. Liver to body weight ratios of control and Yap Tg mice after initial induction of Yap. G. Sox9 staining in high dose AAV-Cre Yap Tg mouse at 2 weeks +Dox comparing PP versus CV areas (100x magnification). Dashed boxes on the left are enlarged in pictures noted on the right. Dashed lines in the PP/CV pictures indicate the borders of the blood vessel. H. Phospho-H3 (pH3) IHC in a TetOYap mouse after 1 week of Yap expression in high dose AAV-Cre model. Periportal (PP) and central venous (CV) areas of the same

mouse are shown. Bar graph represents average pH3 staining per area unit (AU) in PP vs. CV areas. N=3.

Figure S2, related to Figure 2

A. Confocal image (x630) of an 8-week Yap Tg cluster stained for YFP and Vimentin. B. Hepatocyte-specific Yap expression results in upregulation of Sox9 and downregulation of Hnf4α. Representative images of hepatocyte to HPC reprogramming after Yap expression for indicated times. Arrowhead indicates position of weak Sox9 staining.

Figure S4, related to Figure 4

A. Heat map of relative expression of indicated genes in Figure 4B as well as a number of additional markers of hepatocyte phenotype. B. GSEA of control and Yap 6 week cells utilizing MIC1C3+CD133+ gene signature (Dorrell et al., 2011). C. GSEA of control and Yap 6 week cells utilizing gene signature from a model of partial hepatectomy at 24 hours (Singh et al., 2011).

Figure S5, related to Figure 5

A. Schematic representation of liver organoid generation and expansion procedure. Liver pieces were mechanically minced and cultured in 3D matrigel conditions in the presence of R-spondin, Wnt3a, HGF and EGF. After 10 days liver organoid clones were formed, followed by mechanical picking and expansion. B. Microarray gene expression analysis of biliary and hepatocyte markers in primary hepatocytes and wild-type organoids. Orange, green, black and red letterhead represents progenitor-, biliary-, embryonic- and hepatocyte correlated genes respectively. C. GSEA analysis of primary hepatocytes vs. wild-type organoids for a Hippo gene signature (Mohseni et al., 2014). D. Summary analysis of the number of organoids formed per cell number from uninduced (Unind) and induced YAP Tg (OFF DOX and ON DOX) mice. YAP Tg results in dramatic increase of the liver organoid generation in the presence (ON DOX) as well as absence (OFF DOX) of Dox in culture. Total number of plated cells was counted prior plating while number of organoids was counted 10 days after plating. N=5, error bars

represent +/- SEM. E. GSEA analysis of hepatocytes vs. YAP Tg- organoid groups for a previously published HPC gene signature shows significant enrichment in both + and – Dox YAP Tg- organoid groups. F. Microarray gene expression comparison analysis of biliary and hepatoblast markers in primary hepatocytes and +/- Dox YAP Tg organoids. Orange, green and black letterheads represent HPC-, biliary- and hepatoblast correlated genes respectively. G. MTS Proliferation assay of Yap Tg ON DOX single cell YFP+ clone vs. uninduced and wild type cells under monolayer growing conditions. Yap activation results in increased proliferation capacity compared to Yap homeostatic levels. Data represent average of 3 biological replicates +/- SEM. H. Immunostaining analysis of EYFP+ YAP Tg monolayer cells in the presence of Dox (Yap over-expression, ON DOX) demonstrates increased expression of biliary markers (Ck19, Hnf1β and Sox9) and decreased expression of hepatocyte marker Hnf4α compared to cells grown in the absence of Dox (OFF DOX). I. Heat map of 2887 genes grouped by transcriptional gene program using Mclust. Annotated programs of interest are noted to the right of the heat map in bold. Example genes noted as targets of the associated program are listed below the gene program name. J. Fah immunohistochemical analysis of representative intrasplenic cell transplants of primary hepatocyte control cells. Freshly isolated primary hepatocytes were injected into the spleen followed by multiple weight loss/gain cycles via NTBC drug withdrawal/presence respectively. Liver was isolated at weight maintenance state in NTBC OFF stage, sectioned and stained for Fah (positive marker in injected cells) (5X), Hnf4a (hepatocyte marker) and Ck19 (biliary marker). Hematoxylin was used as counterstain.

Figure S6, related to Figure 6

A. ChIP-Seq tracks for TEAD4 from the ENCODE dataset for HepG2 cells in the Notch2/Sox9 loci. Red box below the tracks indicates region where validation PCR product was designed. Graphs to the right depict relative binding of a representative validation experiment (n=3) for Tead4 and Yap in CCLP1 cells. B. Yap/Taz knockdown in CCLP1 cells results in concomitant reduction in *Notch2*/*Sox9* expression as assessed by qPCR. Data represent an average of 3 experiments +/- SEM.

Figure S7, related to Figure 7

A. H&E stain of a representative Yap Tg RBPJ fl/+ and Yap Tg RBPJ fl/fl liver, two weeks after high dose

AAV-Cre and +Dox (100x magnification). B. Genotyping of isolated liver cells from an untreated (-AAV) or

treated (+AAV) RBPJ fl/fl mouse given AAV-cre. RBPJ Δ band appears in hepatocytes, but not biliary

cells after AAV administration. C. RBPJ in situ hybridization/GFP double staining in mice of the indicated

genotypes after treatment with 10^9 pfu/mouse AAV-cre and doxycycline administration for 12 weeks.

Graph to the right indicates number of RBPJ particles/nuclei identified within GFP+ and GFP- areas of the

indicated genotypes (n=3), mean +/- SEM.

Supplemental Experimental Procedures

Mouse Lines, AAV Virus administration, Tamoxifen Induction and YAP Overexpression

Male and female mice were used in this study (except for microarray analysis) and did not show sex-bias differences. AAV-TBG cre (University of Pennsylvania Vector Core) was given to 4 to 8 week old mice retroorbitally. After 3 days, mice were administered doxycycline (1 mg/ml) ad libitum in their cage water. For Ck19-CreERT mice, 4 to 8 week aged mice were given 3 mg of tamoxifen for 5 sequential days. 2 weeks later, doxycycline was started. A minimum of 3 mice were examined per experiment. All mouse procedures and protocols were approved by an AAALAC accredited facility.

Liver organoid differentiation

For liver organoid differentiation of YAP Tg organoids cells were plated on a monolayer of bovine collagen I coated wells (Invitrogen). Confluent cell populations were differentiated using liver organoid growth medium in the presence of 50ng/ml rmEGF, 40ng/ml rmHGF and 1.5uM γ-secretase inhibitor (Calbiochem) and absence of doxycycline. Day 15 differentiated cells were fixed and stained for various hepatocyte and billiary makers.

Cell number counting of liver cells for liver organoid generation

Livers were isolated and digested as described in main text materials and methods. Pelleted cells were re-suspended in media and stained with DAPI (dead cells, Life Technologies) and Calcein blue (live cells). Total number of stained cells were counted using MACSQuant VYB flow cytometer (Miltenyi Biotec) followed by plating under organoid conditions.

Liver organoid infection

For organoid infection using adenovirus, liver organoids were dissociated using a 25 ½ gauge

needle followed by adenoviral infection with spinoculation at MOI=200 in complete liver organoid media for 1hr at 600g at room temperature. Infected cells were then placed at 37°C for 2hrs followed by collection and plating in 3D matrigel conditions. For RBPJ fl/fl R26-stop-YFP organoids AdCre was used for experimental populations while AdFc infected organoids were used as controls; YFP was used as a marker for FACS and microscopic analysis. For Yap/Taz fl/fl organoids, AdCreGFP was used for experimental populations while AdGFP infected organoids served as control cells; GFP was used as a marker for FACS and microscopic analysis. All adenoviruses were purchased from University of Iowa Gene Transfer Vector Core.

Immunohistochemistry, immunofluorescent staining and immunostaining

Antibodies used in this study are noted in **Supplementary Table I**. Tissue was fixed overnight in 10% formalin (Sigma-Aldrich, St. Louis, MO) and embedded in paraffin for sectioning. 5 μM tissue sections were rehydrated followed by antigen retrieval using low pH Antigen Unmasking Solution (Vector Labs, Burlingame, CA). Quenching of endogenous peroxidase and protein block were performed prior to overnight antibody incubation. For immunohistochemistry, Vectastain Elite ABC kit and secondary antibody (Vector Labs) were used to detect primary antibody expression. For immunofluorescence, the Alexa series of fluorescently labeled antibodies (Life Technologies, Grand Island, NY) were used as secondary antibodies at a 1:1000 dilution. For antibodies requiring tyramide amplification, the Vectastain Elite kit was used as directed by the manufacturer with the substitution of the final DAB detection step with the Alexa-488 Tyramide (Life Technologies). Finally, slides were mounted in Prolong Gold (Life Technologies).

For immunostaining, cells in culture were fixed with 4% paraformaldehyde followed by permeabilization with 0.25% Triton X-100 solution. Protein block was performed using 1% BSA solution or serum-free DAKO Protein Block. All primary antibodies were applied for 2hrs at 37°C

followed by 2hr incubation at 37°C with the secondary Alexa series fluorescently labeled antibodies (1:1000 dilution). Prolong Gold antifade reagent with DAPI staining was used for nuclei detection (Life Technologies). For organoid stainings all primary and secondary stainings were performed in suspension followed by cytospins at 500rpm for 5min.

Notch2 Luciferase Assay

The indicated portion of the Notch2 promoter (+750 to +1251 from TSS) was isolated and cloned into the pGL3-Basic vector (Promega). At the two TEA binding sites identified within the Notch2 promoter, three point mutations were generated at each site and this fragment was also cloned into the same vector. CCLP1 cells were co-transfected with a Renilla plasmid and with pCMV-EGFP, pCMV-YAP-S127A (Addgene, Cat# 27370) or pCMV-Yap-5SA (Addgene, Cat #33102). Cells were harvested 72 hours later using the Dual-Glo® Luciferase Assay System (Promega) and assayed according to the manufacturer's directions.

Immunoblotting

Human hepatocyte (Cat# 5206) and biliary (Cat# 5106) lysates were obtained from ScienCell Research Laboratories (Carlsbad, CA). 20 μg of each lysate was run on a polyacrylamide gel and transferred to a PVDF membrane. These were blocked with 5% milk in TBS-T and incubated overnight with the following antibodies, anti-Yap (Cat#4912, Cell Signaling, 1:1000), anti-phosophoYap (Cat#4911, Cell Signaling Technology, 1:1000), anti-CTGF (sc-14939, Santa Cruz Biotechnology, 1:1000), and anti-β-actin (Cat#4970, Cell Signaling Technology, 1:10000).

siRNA knockdown of Yap/Taz in CCLP1 Cells

CCLP1 cell lines were transfected with 5 nM siRNA each (Silencer Select, Ambion) using RNAiMAX (Invitrogen) according to the manufacturers directions. Cells were analyzed approximately 4 days post-transfection. The following oligo pairs were transfected in the

presented experiments, Yap1 (Cat# s20366)/Wwtr1 (Cat# s24787) and Yap1 (Cat# s20368)/ Wwtr1 (Cat# 24789).

Chromatin Immunoprecipitation Assay

Chromatin Immunoprecipitation was performed essentially as previously described (Galli et al., 2012). Briefly, cells were cross-linked in 1% formaldehyde for 10 minutes at room temperature after which the reaction was stopped by addition of 0.125M glycine. Cells were lysed and harvested in ChIP buffer (100 mM Tris at pH 8.6, 0.3% SDS, 1.7% Triton X-100, and 5 mM EDTA) and the chromatin disrupted by sonication using a Diagenode Bioruptor sonicator UCD-200 to obtain fragments of average 200-500 bp in size. Suitable amounts of chromatin were incubated with specific antibodies overnight. Antibodies used were IgG (Sigma, I8140), YAP (a kind gift of Joseph Avruch, MGH, Boston), Tead4 (Abcam Ab58310). Immunoprecipitated complexes were recovered on Protein-A/G agarose beads (Pierce) and, after extensive washes, DNA was recovered by reverse crosslinking and purification using QIAquick PCR purification kit (Qiagen). ChIP-seq tracks for TEAD4 were obtained by uploading publicly available BigWig files from GEO dataset GSE37350 (Home et al., 2012) into mm9 assembly of UCSC genome browser.

  Primer sequences are noted in Supplemental Table 2.

Liver Cell isolation and FACS

All animal studies were approved by the Animal Research Boston Children's Hospital (ARCH). Liver cell isolation was performed via collagenase perfusion as previously described (Klaunig et al., 1981). Briefly, the livers of terminally anesthetized mice given ketamine/xylazine were perfused with Hepatocyte Perfusion Buffer followed by 0.5 mg/ml collagenase (Sigma-Aldrich) in Hepatocyte Digestion Buffer. To maximally liberate biliary cells and other non-parenchymal cells, collagenase-digested livers were then subjected to subsequent serial digestions with Accutase

(EMD Millipore, Billerica, MA) and Trypsin/0.1% EDTA for 20 minutes and 10 minutes, respectively. Cells were collected at each step. Cells were filtered through a 40 μm sieve, washed and stored in PBS with 2% FCS and penicillin/streptomycin prior to sorting. To eliminate non-parenchymal cells, isolated cells were stained with antibodies against CD45 (Cat# 48-0459-42, 1:100, eBiosciences) and Ter119 (Cat# 48-5921, 1:100, eBiosciences) for 30 minutes at 4°C. Biliary cells were enriched by staining and sorting for EpCAM (CD326, Cat#118205, 1:100. BioLegend). YFP+ liver cells were negatively selected for the previously noted antibodies, DAPI staining, and positively sorted for YFP expression.

Hepatocyte Perfusion Buffer

      1x Hanks Balanced Salt Solution

      HEPES, 10 mM

      EDTA, 50 mM

Hepatocyte Digestion Buffer

      1x Hanks Balanced Salt Solution

      HEPES, 10 mM

      EDTA, 50 mM

      Calcium Chloride, 1.25 mM

      Magnesium Chloride, 4 mM

FACS analysis of liver organoids

3D liver organoid matrigel cultures were digested with Dispase (BD Biosciences) for 2hrs at 37°C followed by trypsinization using 0.25% Trypsin (Life Technologies) for 10-15min and spin at 700g/4°C/8min. YFP was used as a marker for RBPJ fl/fl R26-stop-YFP organoids and GFP was used as a marker for Yap/Taz fl/fl organoids. DAPI served as a viability dye. Cells were sorted or analyzed using a FACS Aria III cell sorter (BD Biosciences) or an LSR II flow cytometer (BD Biosciences) respectively.

RNA Recovery, cDNA synthesis, qPCR and Microarray Analysis

Whole liver total RNA was isolated using Trizol Reagent (Life Technologies) according to the manufacter's directions. Total RNA extraction from liver organoids was performed using an RNeasy Mini Kit or miRNeasy Micro Kit (Qiagen). DNase-treated RNA was reversed transcribed using High Capacity RNA to cDNA reagents (Applied Biosystems). qRT-PCR was performed using Fast Taqman reagents (Life Technologies). A full inventory of probes used is available in **Supplementary Table 3**. All reactions were performed using a 1:10 diluted cDNA while mRNA expression levels were estimated using the $2\Delta\Delta CT$ method.

For microarray analysis, total RNA was isolated following FACS using the Qiagen RNeasy Microkit (Qiagen, Valencia, CA) and amplified using the Ovation PicoSL WTA System V2 (Nugen Technologies, San Carlos, CA) according to the manufacturer's directions. The product was then biotin-labeled and fragmented utilizing the Encore Biotin Module (Nugen Technologies) also according to the manufacturer's directions. Labeled sense-strand cDNAs were hybridized to Affymetrix GeneChip Mouse 1.0 ST arrays (Santa Clara, CA).

Microarrays were analyzed using the tools available at the GenePattern site based at the Broad Institute (http://genepattern.broadinstitute.org). Affymetrix CEL files were processed according to the recommended settings at the GenePattern site. The processed and raw data can be found listed under GSE55560 at the Gene Expression Omnibus (GEO). Whole liver arrays (GSM305569, GSM305568, GSM305570) as controls were imported from GEO and utilized as a comparison sample to sorted Yap expressing liver cells. Heat maps were generated from preprocessed GCT files (6536 genes) and arranged according to their Rank Order. For gene set enrichment analysis (GSEA), GCT files containing a total of 34,760 genes were used and processed through the public GSEA server at the Broad Institute.

For enrichment analysis of transcriptionally up/downregulated gene programs, each gene was expressed of as a mixture of normals using Mclust (Fraley and Raftery, 2002) to select the optimal number of clusters. The result of this analysis was the assignment of each gene in each sample to a cluster as annotated in Gene Ontology Biological Process, Reactome, or NetPath. To find genes that had consistent levels of expression within experimental groups, we excluded genes that were assigned to different clusters within the same experimental group, resulting in 1762 genes following 12 distinct patterns of expression across the samples. Overlap between the transcriptional program and each annotation was examined for statistical significance using the Chi-Square test.

Statistical Analysis

A minimum of three separate mice or samples was combined for all displayed graphs unless otherwise indicated. Student's t-test is used to determine significance unless otherwise indicated. Significance is defined as a p value of 0.05 or less. Error bars on all graphs are standard error of the mean or standard deviation.

In situ hybridization (ISH) with GFP staining

ISH for RBPJ transcripts was performed on formalin-fixed liver using RNAscope (ACDBio, Hayward, CA) according to the manufacturer's instructions and developed with the FastRed substrate. After development, slides were then treated for standard immunohistochemistry. Anti-chicken GFP antibody was used to detect GFP expression and slides were processed as described above.

Intrasplenic cell transplantation

All animal studies were approved by the Institutional Animal Care and Use Committee of Boston Children's Hospital. Intrasplenic cell transplantation was performed on recipient FAH mutant mice (Marcus Grompe laboratory). Briefly, mice were anesthetized using ketamine/xylazine. A small flank incision on the left was made to expose the spleen and 1-2 million cells were injected in the spleen blood stream and left to traffic to the liver. The mouse was closed using a two-layered flank wound closure. Injected animals were placed on antibiotics and 75% NTBC drug for 1 day followed by gradual reduction of %NTBC until reach 0% during the period of 4 days. Mice were left off NTBC until their weight was reduced by 15-20% and then placed back on NTBC to regain original weight. The off/on cycle was repeated multiple times until the mouse maintained weight for 1-1 ½ month at off phase followed by euthanasia, liver isolation and fixing with 4% paraformaldehyde. 5uM sections were stained using H&E, FAH, GFP, Hnf4a and Ck19 antibodies.

Proliferation assays

Cells were plated at 5,000 per well cell density in a 96-well plate format and their proliferation kinetics was assessed using CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay (MTS) (Promega) at various time points after plating according to manufacturer instructions.

Supplementary Table I – List of Antibodies for immunocytochemistry

Antibody	Species	Catalog #	Dilution	Manufacturer	Notes
FAH	Rabbit	CLT602-910	1:1000	Cell Lab Tech Inc	
Yap	Rabbit	4912	1:200	Cell Signaling	
Yap	Rabbit		1:800	Gift of Joseph Avruch, MD	
Yap	Mouse	H-69	1:50	Santa Cruz	
Sox9	Rabbit	AB5535	1:1000	Millipore	Tyramide-amplification
Ki67	Rat	M7249	1:50	DakoCytomation	
A6	Rat		1:100	Valentina Factor, NIH	
HNF4α	Goat	sc-6556	1:250	Santa Cruz	
HNF1β	Goat	Sc-	1:250	Santa Cruz	
panCK	Rabbit	Z0622	1:1000	DakoCytomation	
CK19	Rat	Troma-III	1:50	DSHB	
GFP	Rabbit	Ab6556	1:1000	Abcam	
GFP	Chicken	GFP-1020	1:500	Aves Labs	
GFP	Goat	Ab6673	1:100	Abcam	
Phospho-H3	Rabbit	06-570	1:500	Millipore	

Vimentin	Rabbit	2707-1	1:500	Epitomics	
Beta-galactosidase	Rabbit	5863-1	1:100	Epitomics	
Hes1	Rabbit		1:1500	Covance	Tyramide amplification
Jagged1	Goat	AF1277	1:250	R&D Systems	Tyramide amplification

Supplementary Table 2 – List of primer sequences used for ChIP

Target	Primer 1	Primer 2
Pou5f1 negative control (mouse)	GGC AGA CGG CAG ATG CAT AAC	CTC AAT AGC AGA TTA AGG AAG GGC
Ctgf (mouse)	GAA TGT GAG GAA TGT CCC TGT T	CTT GGA GAG AAG AGC TGT GTG A
Sox9 (mouse)	GCA GTG AAA AGA AAT GTC GGA GG	GTT TGT CGT ACT CTC GGA ATG C
Notch2 (mouse)	GCC TCG GAA AGA ATA ACA GCA G	AAA GGT GGA GGC AGG AGA AGT A
β-tubulin negative control (human)	TCC TGT ACC CCC AAG AAC TG	ATT GTT GTC CAT GCT GCA AA
Ctgf (human)	GAG CTG AAT GGA GTC CTA CAC A	GGA GGA ATG CTG AGT GTC AAG
Sox9 (human)	GCT TTT CGA ATA CTG CAA ACT CC	CGC AAG TGT GTG TGT CTA GAC T
Notch2 (human)	CAC ACC AGT TGC CCT ACT TCT C	AGT TTT GAC ACT CCA ACC CCA C

Supplementary Table 3 – List of probes/primers for qRT-PCR

All Taqman probes/primers were purchased from Applied Biosystems

Gene	Catalog number
Afp	Mm00431715_m1
Alb	Mm00802090_m1
Bmi1	Mm03053308_g1
Cd44	Mm01277163_m1
Ctgf	Mm01192933_g1
Cyr61	Mm01323719_g1
GAPDH	4352339E - 1102036
Hes1	Mm01342805_m1
Hhex	Mm00433954_m1
Hnf1β	Mm00447459_m1
Hnf4α	Mm00434964_m1
Jag1	Mm00496902_m1
Jag1	Hs01070032_m1

Ck19	Hs01051611_gH
Lgr5	Mm00438890_m1
Notch1	Mm00435249_m1
Notch1	Hs01062014_m1
Notch2	Mm00803077_m1
Notch2	Hs01050702_m1
Onecut 1	Mm00839394_m1
Sox9	Mm00448840_m1
Sox9	Hs01001343_g1
Taz	Mm01289583_m1
Taz	Hs00210007_m1
Yap	Mm01143263_m1
Yap	Hs00902712_g1

Supplementary Table 4 – List of primers for RBPJ genotyping

Target	Primer 1	Primer 2
RBPJ	TAA CTA TCT TGG AAG GCT AAA AT	AAG AGG GAC ATT GCA TTT TCA CAT
RBPJ Δ	CCT TGG TTT GTT GTT TGG GTT	GTG GCT CTC AAC TCC CAA TCG T
Control	CTA GGC CAC AGA ATT GAA AGA TCT	GTA GGT GGA AAT TCT AGC ATC ATC C

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